

1 **APOLIPOPROTEIN BIOPOLYMER MARKERS**
2 ~~INDICATIVE~~ **-PREDICTIVE OF TYPE II DIABETES**
3

4 FIELD OF THE INVENTION

5 This invention relates to the field of characterizing
6 the existence of a disease state; particularly to the
7 utilization of mass spectrometry to elucidate particular
8 biopolymer markers indicative or predictive of a particular
9 disease state, and most particularly to specific biopolymer
10 markers whose up-regulation, down-regulation, or relative
11 presence in disease vs. normal states has been determined to
12 be useful in disease state assessment and therapeutic target
13 recognition, development and validation.

14 BACKGROUND OF THE INVENTION

15 Methods utilizing mass spectrometry for the analysis of
16 a target polypeptide have been taught wherein the polypeptide
17 is first solubilized in an appropriate solution or reagent
18 system. The type of solution or reagent system, e.g.,
19 comprising an organic or inorganic solvent, will depend on
20 the properties of the polypeptide and the type of mass
21 spectrometry performed and are well-known in the art (see,
22 e.g., Vorm et al. (1994) Anal. Chem. 66:3281 (for MALDI) and
23 Valaskovic et al. (1995) Anal. Chem. 67:3802 (for ESI). Mass
24 spectrometry of peptides is further disclosed, e.g., in WO

1 93/24834 by Chait et al.

2 In one prior art embodiment, the solvent is chosen so
3 that the risk that the molecules may be decomposed by the
4 energy introduced for the vaporization process is
5 considerably reduced, or even fully excluded. This can be
6 achieved by embedding the sample in a matrix, which can be an
7 organic compound, e.g., sugar, in particular pentose or
8 hexose, but also polysaccharides such as cellulose. These
9 compounds are decomposed thermolytically into CO₂ and H₂O so
10 that no residues are formed which might lead to chemical
11 reactions. The matrix can also be an inorganic compound,
12 e.g., nitrate of ammonium which is decomposed practically
13 without leaving any residues. Use of these and other solvents
14 are further disclosed in U.S. Pat. No. 5,062,935 by Schlag et
15 al.

16 Prior art mass spectrometer formats for use in analyzing
17 the translation products include ionization (I) techniques,
18 including but not limited to matrix assisted laser desorption
19 (MALDI), continuous or pulsed electrospray (ESI) and related
20 methods (e.g., IONSPRAY or THERMOSPRAY), or massive cluster
21 impact (MCI); these ion sources can be matched with detection
22 formats including linear or non-linear reflection time-of-
23 flight (TOF), single or multiple quadropole, single or

1 the mass calculation.

2 The mass of the target polypeptide determined by mass
3 spectrometry is then compared to the mass of a reference
4 polypeptide of known identity. In one embodiment, the target
5 polypeptide is a polypeptide containing a number of repeated
6 amino acids directly correlated to the number of
7 trinucleotide repeats transcribed/translated from DNA; from
8 its mass alone the number of repeated trinucleotide repeats
9 in the original DNA which coded it, may be deduced.

10 U.S. Patent No. 6,020,208 utilizes a general category of
11 probe elements (i.e., sample presenting means) with Surfaces
12 Enhanced for Laser Desorption/Ionization (SELDI), within
13 which there are three (3) separate subcategories. The SELDI
14 process is directed toward a sample presenting means (i.e.,
15 probe element surface) with surface-associated (or surface-
16 bound) molecules to promote the attachment (tethering or
17 anchoring) and subsequent detachment of tethered analyte
18 molecules in a light-dependent manner, wherein the said
19 surface molecule(s) are selected from the group consisting of
20 photoactive (photolabile) molecules that participate in the
21 binding (docking, tethering, or crosslinking) of the analyte
22 molecules to the sample presenting means (by covalent
23 attachment mechanisms or otherwise).

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1 PCT/EP/04396 teaches a process for determining the
2 status of an organism by peptide measurement. The reference
3 teaches the measurement of peptides in a sample of the
4 organism which contains both high and low molecular weight
5 peptides and acts as an indicator of the organism's status.
6 The reference concentrates on the measurement of low
7 molecular weight peptides, i.e. below 30,000 Daltons, whose
8 distribution serves as a representative cross-section of
9 defined controls. Contrary to the methodology of the instant
10 invention, the '396 patent strives to determine the status of
11 a healthy organism, i.e. a "normal" and then use this as a
12 reference to differentiate disease states. The present
13 inventors do not attempt to develop a reference "normal", but
14 rather strive to specify particular markers whose presence,
15 absence or relative strength/concentration in disease vs.
16 normal is diagnostic of at least one specific disease state
17 or whose up-regulation or down-regulation is predictive of at
18 least one specific disease state, whereby the presence of
19 said marker serves as a positive indicator useful in
20 distinguishing disease state. This leads to a simple method
21 of analysis which can easily be performed by an untrained
22 individual, since there is a positive correlation of data.
23 On the contrary, the '396 patent requires a complicated

1 analysis by a highly trained individual to determine disease
2 state versus the perception of non-disease or normal
3 physiology.

4 Richter et al, Journal of Chromatography B, 726(1999)
5 25-35, refer to a database established from human
6 hemofiltrate comprised of a mass database and a sequence
7 database. The goal of Richter et al was to analyze the
8 composition of the peptide fraction in human blood. Using
9 MALDI-TOF, over 20,000 molecular masses were detected
10 representing an estimated 5,000 different peptides. The
11 conclusion of the study was that the hemofiltrate (HF)
12 represented the peptide composition of plasma. No
13 correlation of peptides with relation to normal and/or
14 disease states is made.

15 As used herein, "analyte" refers to any atom and/or
16 molecule; including their complexes and fragment ions. The
17 term may refer to a single component or a set of components.
18 In the case of biological molecules/macromolecules or
19 "biopolymers", such analytes include but are not limited to:
20 polypeptides, polynucleotides, proteins, peptides,
21 antibodies, DNA, RNA, carbohydrates, steroids, and lipids,
22 and any detectable moiety thereof, e.g. immunologically
23 detectable fragments. Note that most important biomolecules

1 under investigation for their involvement in the structure or
2 regulation of life processes are quite large (typically
3 several thousand times larger than H₂O).

4 As used herein, the term "molecular ions" refers to
5 molecules in the charged or ionized state, typically by the
6 addition or loss of one or more protons (H⁺).

7 As used herein, the term "molecular fragmentation" or
8 "fragment ions" refers to breakdown products of analyte
9 molecules caused, for example, during laser-induced
10 desorption (especially in the absence of added matrix).

11 As used herein, the term "solid phase" refers to the
12 condition of being in the solid state, for example, on the
13 probe element surface.

14 As used herein, "gas" or "vapor phase" refers to
15 molecules in the gaseous state (i.e., in vacuo for mass
16 spectrometry).

17 As used herein, the term "analyte desorption/ionization"
18 refers to the transition of analytes from the solid phase to
19 the gas phase as ions. Note that the successful
20 desorption/ionization of large, intact molecular ions by
21 laser desorption is relatively recent (circa 1988)--the big
22 breakthrough was the chance discovery of an appropriate
23 matrix (nicotinic acid).

1 As used herein, the term "gas phase molecular ions"
2 refers to those ions that enter into the gas phase. Note that
3 large molecular mass ions such as proteins (typical
4 mass=60,000 to 70,000 times the mass of a single proton) are
5 typically not volatile (i.e., they do not normally enter into
6 the gas or vapor phase). However, in the procedure of the
7 present invention, large molecular mass ions such as proteins
8 do enter the gas or vapor phase.

9 As used herein in the case of MALDI, the term "matrix"
10 refers to any one of several small, acidic, light absorbing
11 chemicals (e.g., CHCA (alpha-cyano-4-hydroxy-cinnamic acid),
12 nicotinic or sinapinic acid) that is mixed in solution with
13 the analyte in such a manner so that, upon drying on the
14 probe element, the crystalline matrix-embedded analyte
15 molecules are successfully desorbed (by laser irradiation)
16 and ionized from the solid phase (crystals) into the gaseous
17 or vapor phase and accelerated as intact molecular ions. For
18 the MALDI process to be successful, analyte is mixed with a
19 freshly prepared solution of the chemical matrix (e.g.,
20 10,000:1 matrix:analyte) and placed on the inert probe
21 element surface to air dry just before the mass spectrometric
22 analysis. The large fold molar excess of matrix, present at
23 concentrations near saturation, facilitates crystal formation

1 and entrapment of analyte.

2 As used herein, "energy absorbing molecules (EAM)"
3 refers to any one of several small, light absorbing chemicals
4 that, when presented on the surface of a probe, facilitate
5 the neat desorption of molecules from the solid phase (i.e.,
6 surface) into the gaseous or vapor phase for subsequent
7 acceleration as intact molecular ions. The term EAM is
8 preferred, especially in reference to SELDI. Note that
9 analyte desorption by the SELDI process is defined as a
10 surface-dependent process (i.e., neat analyte may be placed
11 on a surface composed of bound EAM or EAM and analyte may be
12 mixed prior to placement on a surface). In contrast, MALDI is
13 presently thought to facilitate analyte desorption by a
14 volcanic eruption-type process that "throws" the entire
15 surface into the gas phase. Furthermore, note that some EAM
16 when used as free chemicals to embed analyte molecules as
17 described for the MALDI process will not work (i.e., they do
18 not promote molecular desorption, thus they are not suitable
19 matrix molecules).

20 As used herein, "probe element" or "sample presenting
21 device" refers to an element having the following properties:
22 it is inert (for example, typically stainless steel) and
23 active (probe elements with surfaces enhanced to contain EAM

1 and/or molecular capture devices).

2 As used herein, "MALDI" refers to Matrix-Assisted Laser
3 Desorption/Ionization.

4 As used herein, "TOF" stands for Time-of-Flight.

5 As used herein, "MS" refers to Mass Spectrometry.

6 As used herein, "MS/MS" refers to multiple sequential
7 mass spectrometry.

8 As used herein "MALDI-TOF MS" refers to Matrix-assisted
9 laser desorption/ionization time-of-flight mass spectrometry.

10 As used herein, "ESI" is an abbreviation for
11 electrospray ionization.

12 As used herein, "chemical bonds" is used simply as an
13 attempt to distinguish a rational, deliberate, and
14 knowledgeable manipulation of known classes of chemical
15 interactions from the poorly defined kind of general
16 adherence observed when one chemical substance (e.g., matrix)
17 is placed on another substance (e.g., an inert probe element
18 surface). Types of defined chemical bonds include
19 electrostatic or ionic (+/-) bonds (e.g., between a
20 positively and negatively charged groups on a protein
21 surface), covalent bonds (very strong or "permanent" bonds
22 resulting from true electron sharing), coordinate covalent
23 bonds (e.g., between electron donor groups in proteins and

1 transition metal ions such as copper or iron), and
2 hydrophobic interactions (such as between two noncharged
3 groups), weak dipole and London force or induced dipole
4 interactions.

5 As used herein, "electron donor groups" refers to the
6 case of biochemistry, where atoms in biomolecules (e.g, N, S,
7 O) "donate" or share electrons with electron poor groups
8 (e.g., Cu ions and other transition metal ions).

9 As used herein, the term "biopolymer markers indicative
10 or predictive of a disease state" is interpreted to mean that
11 a biopolymer marker which is strongly present in a normal
12 individual, but is down-regulated in disease is predictive of
13 said disease; while alternatively, a biopolymer marker which
14 is strongly present in a disease state, but is down-regulated
15 in normal individuals, is indicative of said disease state.
16 Biopolymer markers which are present in both disease and
17 normal states are indicative/predictive based upon their
18 relative strengths in disease vs. normal, along with the
19 observation regarding when their signal strengthens/weakens
20 relative to disease manifestation or progression.

21 As used herein, the term "disease state assessment" is
22 interpreted to mean quantitative or qualitative determination
23 of the presence/absence of the disease, with or without an

1 ability to determine severity, rapidity of onset, or
2 resolution of the disease state, e.g. a return to a normal
3 physiological state.

4 As used herein, the term "therapeutic target
5 recognition, development, and validation" refers to any
6 concept or method which enables an artisan to recognize,
7 develop, or validate the efficacy of a therapeutic moiety
8 which is effected in conjunction with a chemical or physical
9 interaction with one or more of the biopolymer markers of the
10 instant invention.

11 As used herein, the term "polypeptide" is interpreted to
12 mean a polymer composed of amino acid residues, related
13 naturally occurring structural variants, and synthetic non-
14 naturally occurring analogs thereof linked via peptide bonds,
15 related naturally occurring structural variants, and
16 synthetic non-naturally occurring analogs thereof. Synthetic
17 polypeptides can be synthesized, for example, using an
18 automated polypeptide synthesizer. The term "protein"
19 typically refers to large polypeptides. The term "peptide"
20 typically refers to short polypeptides. "Polypeptide(s)"
21 refers to any peptide or protein comprising two or more amino
22 acids joined to each other by peptide bonds or modified
23 peptide bonds. "Polypeptide(s)" refers to both short chains,

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commonly referred to as peptides, oligopeptides and oligomers
and to longer chains generally referred to as proteins.
Polypeptides may contain amino acids other than the 20 gene
encoded amino acids. "Polypeptide(s)" include those modified
either by natural processes, such as processing and other
post-translational modifications, but also by chemical
modification techniques. Such modifications are well
described in basic texts and in more detailed monographs, as
well as in a voluminous research literature, and they are
well-known to those of skill in the art. It will be
appreciated that the same type of modification may be present
in the same or varying degree at several sites in a given
polypeptide. Also, a given polypeptide may contain many types
of modifications. Modifications can occur anywhere in a
polypeptide, including the peptide backbone, the amino acid
side-chains, and the amino or carboxyl termini. Modifications
include, for example, acetylation, acylation, ADP-
ribosylation, amidation, covalent attachment of flavin,
covalent attachment of a heme moiety, covalent attachment of
a nucleotide or nucleotide derivative, covalent attachment of
a lipid or lipid derivative, covalent attachment of
phosphatidylinositol, cross-linking, cyclization, disulfide
bond formation, demethylation, formation of covalent cross-

1 links, formation of cysteine, formation of pyroglutamate,
 2 formylation, gamma-carboxylation, glycosylation, GPI anchor
 3 formation, hydroxylation, iodination, methylation,
 4 myristoylation, oxidation, proteolytic processing,
 5 phosphorylation, prenylation, racemization, glycosylation,
 6 lipid attachment, sulfation, gamma-carboxylation of glutamic
 7 acid residues, hydroxylation and ADP-ribosylation,
 8 selenoylation, sulfation, transfer-RNA mediated addition of
 9 amino acids to proteins, such as arginylation, and
 10 ubiquitination. See, for instance, PROTEINS--STRUCTURE AND
 11 MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman
 12 and Company, New York (1993) and Wold, F., Posttranslational
 13 Protein Modifications: Perspectives and Prospects, pgs. 1-12
 14 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C.
 15 Johnson, Ed., Academic Press, New York (1983); Seifter et
 16 al., Meth. Enzymol. 182:626-646 (1990) and Rattan et al.,
 17 Protein Synthesis: Posttranslational Modifications and Aging,
 18 Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be
 19 branched or cyclic, with or without branching. Cyclic,
 20 branched and branched circular polypeptides may result from
 21 post-translational natural processes and may be made by
 22 entirely synthetic methods, as well.

23 As used herein, the term "polynucleotide" is interpreted

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1 to mean a polymer composed of nucleotide units.

2 Polynucleotides include naturally occurring nucleic acids,
3 such as deoxyribonucleic acid ("DNA") and ribonucleic acid
4 ("RNA") as well as nucleic acid analogs. Nucleic acid analogs
5 include those which include non-naturally occurring bases,
6 nucleotides that engage in linkages with other nucleotides
7 other than the naturally occurring phosphodiester bond or
8 which include bases attached through linkages other than
9 phosphodiester bonds. Thus, nucleotide analogs include, for
10 example and without limitation, phosphorothioates,
11 phosphorodithioates, phosphorotriesters,
12 phosphoramidates, boranophosphates, methylphosphonates,
13 chiral-methyl phosphonates, 2-O-methyl ribonucleotides,
14 peptide-nucleic acids (PNAs), and the like. Such
15 polynucleotides can be synthesized, for example, using an
16 automated DNA synthesizer. The term "nucleic acid" typically
17 refers to large polynucleotides. The term "oligonucleotide"
18 typically refers to short polynucleotides, generally no
19 greater than about 50 nucleotides. It will be understood that
20 when a nucleotide sequence is represented by a DNA sequence
21 (i.e., A, T, G, C), this also includes an RNA sequence (i.e.,
22 A, U, G, C) in which "U" replaces T.

23 As used herein, the term "detectable moiety" or a

"label" refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P , ^{35}S , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin-streptavidin, dioxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The detectable moiety often generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantitate the amount of bound detectable moiety in a sample. The detectable moiety can be incorporated in or attached to a primer or probe either covalently, or through ionic, van der Waals or hydrogen bonds, e.g., incorporation of radioactive nucleotides, or biotinylated nucleotides that are recognized by streptavidin. The detectable moiety may be directly or indirectly detectable. Indirect detection can involve the binding of a second directly or indirectly detectable moiety to the detectable moiety. For example, the detectable moiety can be the ligand of a binding partner, such as biotin, which is a binding partner for streptavidin, or a nucleotide sequence, which is the binding partner for a complementary sequence, to which it can specifically

1 hybridize. The binding partner may itself be directly
2 detectable, for example, an antibody may be itself labeled
3 with a fluorescent molecule. The binding partner also may be
4 indirectly detectable, for example, a nucleic acid having a
5 complementary nucleotide sequence can be a part of a branched
6 DNA molecule that is in turn detectable through hybridization
7 with other labeled nucleic acid molecules. (See, e.g., P. D.
8 Fahrlander and A. Klausner, Bio/Technology (1988) 6:1165.)
9 Quantitation of the signal is achieved by, e.g.,
10 scintillation counting, densitometry, or flow cytometry.

11 As used herein, the term "antibody or antibodies"
12 includes polyclonal and monoclonal antibodies of any isotype
13 (IgA, IgG, IgE, IgD, IgM), or an antigen-binding portion
14 thereof, including but not limited to F(ab) and Fv fragments,
15 single chain antibodies, chimeric antibodies, humanized
16 antibodies, and a Fab expression library. "Antibody" refers
17 to a polypeptide ligand substantially encoded by an
18 immunoglobulin gene or immunoglobulin genes, or fragments
19 thereof, which specifically binds and recognizes an epitope
20 (e.g., an antigen). The recognized immunoglobulin --genes
21 include the kappa and lambda light chain constant region
22 genes, the alpha, gamma, delta, epsilon and mu heavy chain
23 constant region genes, and the myriad immunoglobulin variable

1 region genes. Antibodies exist, e.g., as intact
2 immunoglobulins or as a number of well characterized
3 fragments produced by digestion with various peptidases. This
4 includes, e.g., Fab' and F(ab)'₂ fragments. The term
5 "antibody," as used herein, also includes antibody fragments
6 either produced by the modification of whole antibodies or
7 those synthesized de novo using recombinant DNA
8 methodologies. It also includes polyclonal antibodies,
9 monoclonal antibodies, chimeric antibodies and humanized
10 antibodies. "Fc" portion of an antibody refers to that
11 portion of an immunoglobulin heavy chain that comprises one
12 or more heavy chain constant region domains, CH, CH₂ and CH₃,
13 but does not include the heavy chain variable region.

14 As used herein, the term "moieties" refers to an
15 indefinite portion of a sample.

16 A "ligand" is a compound that specifically binds to a
17 target molecule.

18 A "receptor" is a compound or portion of a structure
19 that specifically binds to a ligand.

20 A ligand or a receptor (e.g., an antibody) "specifically
21 binds to" or "is specifically immunoreactive with" a compound
22 analyte when the ligand or receptor functions in a binding
23 reaction which is determinative of the presence of the

1 plants, fungi, or animal cells or tissues. Suitable
2 techniques involve selection of libraries of recombinant
3 antibodies in phage or similar vectors. See, Huse et al.
4 (1989) Science 246: 1275-1281; and Ward et al. (1989) Nature
5 341: 544-546. The protocol described by Huse is
6 rendered more efficient in combination with phage display
7 technology. See, e.g., Dower et al., WO 91/17271 and
8 McCafferty et al., WO 92/01047.

9 As used herein, the term "isolated" is interpreted to
10 mean altered "by the hand of man" from its natural state,
11 i.e., if it occurs in nature, it has been changed or removed
12 from its original environment, or both. For example, a
13 polynucleotide or a polypeptide naturally present in a living
14 organism is not "isolated," but the same polynucleotide or
15 polypeptide separated from the coexisting materials of its
16 natural state is "isolated", as the term is employed herein.

17 As used herein, the term "variant" is interpreted to
18 mean a polynucleotide or polypeptide that differs from a
19 reference polynucleotide or polypeptide respectively, but
20 retains essential properties. A typical variant of a
21 polynucleotide differs in nucleotide sequence from another,
22 reference polynucleotide. Changes in the nucleotide sequence
23 of the variant may or may not alter the amino acid sequence

1 of a polypeptide encoded by the reference polynucleotide.
2 Nucleotide changes may result in amino acid substitutions,
3 additions, deletions, fusions and truncations in the
4 polypeptide encoded by the reference sequence, as discussed
5 below. A typical variant of a polypeptide differs in amino
6 acid sequence from another, reference polypeptide. Generally,
7 differences are limited so that the sequences of the
8 reference polypeptide and the variant are closely similar
9 overall and, in many regions, identical. A variant and
10 reference polypeptide may differ in amino acid sequence by
11 one or more substitutions, additions, deletions in any
12 combination. A substituted or inserted amino acid residue may
13 or may not be one encoded by the genetic code. A variant of a
14 polynucleotide or polypeptide may be a naturally occurring
15 such as an allelic variant, or it may be a variant that is
16 not known to occur naturally. Non-naturally occurring
17 variants of polynucleotides and polypeptides may be made by
18 mutagenesis techniques, by direct synthesis, and by other
19 recombinant methods known to skilled artisans.

20 As used herein, the term "biopolymer marker" refers to a
21 polymer of biological origin, e.g. polypeptides,
22 polynucleotides, polysaccharides or polyglycerides (e.g., di-
23 or tri-glycerides), and may include any fragment, e.g.

1 immunologically reactive fragments, variants or moieties
2 thereof.

3 As used herein, the term "fragment" refers to the
4 products of the chemical, enzymatic, or physical breakdown of
5 an analyte. Fragments may be in a neutral or ionic state.

6 As used herein, the term "therapeutic avenues" is
7 interpreted to mean any agents, modalities, synthesized
8 compounds, etc., which interact with a biopolymer marker in
9 any manner that facilitates a therapeutic benefit, including
10 immunotherapeutic intervention, e.g. modalities such as
11 administration of an immunologically reactive moiety capable
12 of altering the course, progression and/or manifestation of
13 the disease, as a result of interfering with the disease
14 manifestation process, for example, at the early stages
15 focused upon by the identification of the disease, such as by
16 supplying a moiety capable of modifying the pathogenicity of
17 lymphocytes specific for the biopolymer marker or related
18 components.

19 As used herein, the term "interacting with a biopolymer
20 marker" includes any process by which a biopolymer marker may
21 physically or chemically relate with an organism,
22 particularly when this interaction results in the development
23 of therapeutic avenues or in modulation of the disease state.

1 As used herein, the term "therapeutic targets" may thus
2 be defined as those analytes which are capable of exerting a
3 modulating force, wherein "modulation" is defined as an
4 alteration in function inclusive of activity, synthesis,
5 production, and circulating levels. Thus, modulation effects
6 the level or physiological activity of at least one
7 particular disease related biopolymer marker or any compound
8 or biomolecule whose presence, level or activity is linked
9 either directly or indirectly, to an alteration of the
10 presence, level, activity or generic function of the
11 biopolymer marker, and may include pharmaceutical agents,
12 biomolecules that bind to the biopolymer markers, or
13 biomolecules or complexes to which the biopolymer markers
14 bind. The binding of the biopolymer markers and the
15 therapeutic moiety may result in activation (agonist),
16 inhibition (antagonist), or an increase or decrease in
17 activity or production (modulator) of the biopolymer markers
18 or the bound moiety. Examples of such therapeutic moieties
19 include, but are not limited to, antibodies,
20 oligonucleotides, proteins (e.g., receptors), RNA, DNA,
21 enzymes, peptides or small molecules. With regard to
22 immunotherapeutic moieties, such a moiety may be defined as
23 an effective analog for a major epitope peptide which has the

1 ability to reduce the pathogenicity of key lymphocytes which
2 are specific for the native epitope. An analog is defined as
3 having structural similarity but not identity in peptide
4 sequencing able to be recognized by T-cells spontaneously
5 arising and targeting the endogeneous self epitope. A
6 critical function of this analog is an altered T-cell
7 activation which leads to T-cell anergy or death.

8 With the advent of mass spectrometric methods such as
9 MALDI and SELDI and ESI, researchers have begun to utilize a
10 tool that holds the promise of uncovering countless
11 biopolymers which result from translation, transcription and
12 post-translational transcription of proteins from the entire
13 genome.

14 Operating upon the principles of retentate
15 chromatography, SELDI MS involves the adsorption of proteins,
16 based upon their physico-chemical properties at a given pH
17 and salt concentration, followed by selectively desorbing
18 proteins from the surface by varying pH, salt, or organic
19 solvent concentration. After selective desorption, the
20 proteins retained on the SELDI surface, the "chip", can be
21 analyzed using the CIPHERGEN protein detection system, or an
22 equivalent thereof. Retentate chromatography is limited,
23 however, by the fact that if unfractionated body fluids, e.g.

1 blood, blood products, urine, saliva, cerebrospinal fluid,
2 lymph and the like, along with tissue samples, are applied
3 to the adsorbent surfaces, the biopolymers present in the
4 greatest abundance will compete for all the available binding
5 sites and thereby prevent or preclude less abundant
6 biopolymers from interacting with them, thereby reducing or
7 eliminating the diversity of biopolymers which are readily
8 ascertainable.

9 If a process could be devised for maximizing the
10 diversity of biopolymers discernable from a sample, the
11 ability of researchers to accurately determine the relevance
12 of such biopolymers with relation to one or more disease
13 states would be immeasurably enhanced.

14
15 SUMMARY OF THE INVENTION

16 The instant invention is characterized by the use of a
17 combination of preparatory steps, e.g. chromatography and 1-D
18 tricine polyacrylamide gel electrophoresis. Subsequent to
19 which the gel is stained, e.g. with Coomassie blue, silver or
20 rubidium. Next, bands are selected from the gels for further
21 study. Tryptic digestion of each band follows, concluding
22 with the extraction of tryptic peptides from the digest.
23 This extraction may be accomplished utilizing C18 ZIPTIPs, or

1 organic extract and dry technique followed by MALDI Qq TOF
2 (Maldi Quadrupole Quadrupole Time of Flight) processing.

3 Additional methodologies may include SELDI MS, 2-D gel
4 technology, MALDI MS/MS and time-of-flight detection
5 procedures to maximize the diversity of biopolymers which are
6 verifiable within a particular sample. The cohort of
7 biopolymers verified within a sample is then compared to
8 develop data indicating their presence, absence or relative
9 strength/concentration in disease vs normal controls, and
10 further studied to determine whether the up-regulation or
11 down-regulation of a single biopolymer or group of
12 biopolymers is indicative of a disease state or predictive of
13 the development of said disease state. Additionally,
14 biopolymers recognized as being indicative or predictive of a
15 disease state in accordance with the instant invention are
16 useful in therapeutic intervention, e.g. as therapeutic
17 modalities in their own right, in the course of therapeutic
18 target recognition, in the development and validation of
19 efficacious therapeutic modalities, e.g when interrogating or
20 developing phage display libraries, and as ligands or
21 receptors for use in conjunction with therapeutic
22 intervention.

23 Although all manner of biomarkers related to all disease

1 conditions are deemed to be within the purview of the instant
2 invention and methodology, particular significance was given
3 to those markers and diseases associated with the complement
4 system, cognitive diseases, e.g. Alzheimer's disease and
5 Syndrome X and diseases related thereto.

6 The complement system is an important part of non-clonal
7 or innate immunity that collaborates with acquired immunity
8 to destroy invading pathogens and to facilitate the clearance
9 of immune complexes from the system. This system is the
10 major effector of the humoral branch of the immune system,
11 consisting of nearly 30 serum and membrane proteins. The
12 proteins and glycoproteins composing the complement system
13 are synthesized largely by liver hepatocytes. Activation of
14 the complement system involves a sequential enzyme cascade in
15 which the proenzyme product of one step becomes the enzyme
16 catalyst of the next step. Complement activation can occur
17 via two pathways: the classical and the alternative. The
18 classical pathway is commonly initiated by the formation of
19 soluble antigen-antibody complexes or by the binding of
20 antibody to antigen on a suitable target, such as a bacterial
21 cell. The alternative pathway is generally initiated by
22 various cell-surface constituents that are foreign to the
23 host. Each complement component is designated by numerals

1 (C1-C9), by letter symbols, or by trivial names. After a
2 component is activated, the peptide fragments are denoted by
3 small letters. The complement fragments interact with one
4 another to form functional complexes. Ultimately, foreign
5 cells are destroyed through the process of a membrane-attack
6 complex mediated lysis.

7 The C4 component of the complement system is involved in
8 the classical activation pathway. It is a glycoprotein
9 containing three polypeptide chains (α , β , and γ). C4 is a
10 substrate of component C1s and is activated when C1s
11 hydrolyzes a small fragment (C4a) from the amino terminus of
12 the α chain, exposing a binding site on the larger fragment
13 (C4b).

14 The native C3 component consists of two polypeptide
15 chains, α and β . As a serum protein, C3 is involved in the
16 alternative pathway. Serum C3, which contains an unstable
17 thioester bond, is subject to slow spontaneous hydrolysis
18 into C3a and C3b. The C3f component is involved in the
19 regulation required of the complement system which confines
20 the reaction to designated targets. During the regulation
21 process, C3b is cleaved into two parts: C3bi and C3f. C3bi
22 is a membrane-bound intermediate wherein C3f is a free
23 diffusible (soluble) component.

1 Complement components have been implicated in the
2 pathogenesis of several disease conditions. C3 deficiencies
3 have the most severe clinical manifestations, such as
4 recurrent bacterial infections and immune-complex diseases,
5 reflecting the central role of C3. The rapid profusion of
6 C3f moieties and resultant "accidental" lysis of normal cells
7 mediated thereby gives rise to a host of auto-immune
8 reactions. The ability to understand and control these
9 mechanisms, along with their attendant consequences, will
10 enable practitioners to develop both diagnostic and
11 therapeutic avenues by which to thwart these maladies.

12 In the course of defining a plurality of disease
13 specific marker sequences, special significance was given to
14 markers which were evidentiary of a particular disease state
15 or with conditions associated with Syndrome-X. Syndrome-X is
16 a multifaceted syndrome, which occurs frequently in the
17 general population. A large segment of the adult population
18 of industrialized countries develops this metabolic syndrome,
19 produced by genetic, hormonal and lifestyle factors such as
20 obesity, physical inactivity and certain nutrient excesses.
21 This disease is characterized by the clustering of insulin
22 resistance and hyperinsulinemia, and is often associated with
23 dyslipidemia (atherogenic plasma lipid profile), essential

1 hypertension, abdominal (visceral) obesity, glucose
2 intolerance or noninsulin-dependent diabetes mellitus and an
3 increased risk of cardiovascular events. Abnormalities of
4 blood coagulation (higher plasminogen activator inhibitor
5 type I and fibrinogen levels), hyperuricemia and
6 microalbuminuria have also been found in metabolic syndrome-
7 X.

8 The instant inventors view the Syndrome X continuum in
9 its cardiovascular light, while acknowledging its important
10 metabolic component. The first stage of Syndrome X consists
11 of insulin resistance, abnormal blood lipids (cholesterol,
12 triglycerides and free fatty acids), obesity, and high blood
13 pressure (hypertension). Any one of these four first stage
14 conditions signals the start of Syndrome X.

15 Each first stage Syndrome X condition risks leading to
16 another. For example, increased insulin production is
17 associated with high blood fat levels, high blood pressure,
18 and obesity. Furthermore, the effects of the first stage
19 conditions are additive; an increase in the number of
20 conditions causes an increase in the risk of developing more
21 serious diseases on the Syndrome X continuum.

22 A patient who begins the Syndrome X continuum risks
23 spiraling into a maze of increasingly deadly diseases. The

1 next stages of the Syndrome X continuum lead to overt
2 diabetes, kidney failure, and heart failure, with the
3 possibility of stroke and heart attack at any time. Syndrome
4 X is a dangerous continuum, and preventative medicine is the
5 best defense. Diseases are currently most easily diagnosed
6 in their later stages, but controlling them at a late stage
7 is extremely difficult. Disease prevention is much more
8 effective at an earlier stage.

9 In a further contemplated embodiment of the invention,
10 samples may be taken from a patient at one point in time, as
11 a single sample or as multiple samples, or at different
12 points in time such that analysis is carried out on multiple
13 samples for ongoing analysis. Typically, a first sample is
14 taken from a patient upon presentation with possible symptoms
15 of a disease and analyzed according to the invention.
16 Subsequently, some period of time after presentation, for
17 example, about 3 - 6 months after the first presentation, a
18 second sample is taken and analyzed according to the
19 invention. The data can be used, by way of example, to
20 diagnose or monitor a disease state, determine risk
21 assessment, identify therapeutic avenues, or determine the
22 therapeutic value of an agent such as a pharmaceutical.

23 Subsequent to the isolation of particular disease state

1 marker sequences as taught by the instant invention, the
2 promulgation of various forms of risk assessment tests are
3 contemplated which will allow physicians to identify
4 asymptomatic patients before they suffer an irreversible
5 event such as diabetes, kidney failure, and heart failure,
6 and enable effective disease management and preventative
7 medicine. Additionally, the specific diagnostic tests which
8 evolve from this methodology provide a tool for rapidly and
9 accurately diagnosing acute Syndrome X events such as heart
10 attack and stroke, and facilitate treatment.

11 More particularly, biopolymer markers elucidated via
12 methodologies of the instant invention find utility related
13 to broad areas of disease therapeutics. Such therapeutic
14 avenues include, but are not limited to:

15 1) utilization and recognition of said biopolymer
16 markers, variants or moieties thereof as direct therapeutic
17 modalities, either alone or in conjunction with an effective
18 amount of a pharmaceutically effective carrier;

19 2) validation of therapeutic modalities or disease
20 preventative agents as a function of biopolymer marker
21 presence or concentration;

22 3) treatment or prevention of a disease state by
23 formation of disease intervention modalities; e.g. formation

1 of biopolymer/ligand conjugates which intervene at receptor
2 sites to prevent, delay or reverse a disease process;

3 4) use of biopolymer markers or moieties thereof as a
4 means of elucidating therapeutically viable agents, e.g. from
5 a bacteriophage peptide display library, a bacteriophage
6 antibody library or the like;

7 5) instigation of a therapeutic immunological
8 response; and

9 6) synthesis of molecular structures related to said
10 biopolymer markers, moieties or variants thereof which are
11 constructed and arranged to therapeutically intervene in the
12 disease process.

13 A process for identifying or developing therapeutic
14 avenues related to a disease state utilizing any of the above
15 examples may follow results obtained from conducting an
16 analysis inclusive of interacting with a biopolymer including
17 the sequence of the particular disease specific marker or at
18 least one analyte thereof of the present invention. Such
19 treatment or prevention of a disease state by formation of
20 disease intervention modalities may be by the formation of
21 biopolymer/ligand conjugates which intervene at receptor
22 sites to prevent, delay, or reverse a disease process. In
23 addition, a means of elucidating therapeutically viable

agents may include the use of a bacteriophage peptide display library or a bacteriophage antibody library. The therapeutic avenues may regulate the presence or absence of the biopolymer including the sequence of the particular disease specific marker or at least one analyte thereof in the present invention.

Accordingly, it is an objective of the instant invention to define a disease specific biopolymer marker sequence which is useful in evidencing and categorizing at least one particular disease state.

It is an additional objective of the instant invention to develop methods and means of disease therapy, including but not limited to:

1) utilization and recognition of said biopolymer markers, variants or moieties thereof as direct therapeutic modalities, either alone or in conjunction with an effective amount of a pharmaceutically effective carrier;

2) validation of therapeutic modalities or disease preventative agents as a function of biopolymer marker presence or concentration;

3) treatment or prevention of a disease state by formation of disease intervention modalities; e.g. formation of biopolymer/ligand conjugates which intervene at receptor

1 sites to prevent, delay or reverse a disease process;

2 4) use of biopolymer markers or moieties thereof as a
3 means of elucidating therapeutically viable agents, e.g. from
4 a bacteriophage peptide display library, a bacteriophage
5 antibody library or the like;

6 5) instigation of a therapeutic immunological
7 response; and

8 6) synthesis of molecular structures related to said
9 biopolymer markers, moieties or variants thereof which are
10 constructed and arranged to therapeutically intervene in the
11 disease process, e.g. by directly determining the three-
12 dimensional structure of said biopolymer marker directly from
13 an amino acid sequence thereof.

14 It is another objective of the instant invention to
15 evaluate samples containing a plurality of biopolymers for
16 the presence of disease specific biopolymer marker sequences
17 (disease specific markers) which evidence a link to at least
18 one specific disease state.

19 It is a further objective of the instant invention to
20 elucidate essentially all biopolymeric markers, moieties or
21 variants thereof contained within said samples, whereby
22 particularly significant moieties may be identified.

23 It is a further objective of the instant invention

1 provide at least one purified antibody which is specific to
2 said disease specific marker sequence.

3 It is yet another objective of the instant invention to
4 teach a monoclonal antibody which is specific to said disease
5 specific marker sequence.

6 It is a still further objective of the invention to
7 teach polyclonal antibodies raised against said disease
8 specific marker.

9 It is yet an additional objective of the instant
10 invention to teach a diagnostic kit for determining the
11 presence, concentration, or relative strength/concentration
12 of said disease specific marker.

13 It is a still further objective of the instant invention
14 to teach methods for characterizing disease state based upon
15 the identification of said disease specific marker.

16 Other objects and advantages of this invention will
17 become apparent from the following description taken in
18 conjunction with the accompanying drawings wherein are set
19 forth, by way of illustration and example, certain
20 embodiments of this invention. The drawings constitute a
21 part of this specification and include exemplary embodiments
22 of the present invention and illustrate various objects and
23 features thereof.

1

2 BRIEF DESCRIPTION OF THE FIGURES

3 Figure 1 is a photograph of a tricine gel HiQ 3 (scrub)

4 comparing Type II Diabetes versus a normal specimen;

5 Figure 2 is a trypsin digested spectra graph depicting the

6 ion 1353; and

7 Figure 3 is a trypsin digested spectra graph depicting the

8 ion 1104.

9

10 DETAILED DESCRIPTION OF THE INVENTION

11 In earlier work, for example in U.S. Patent application

12 09/846330 filed April 30, 2000, the contents of which is

13 herein incorporated by reference, raw sera was obtained and

14 mixed with formic acid and extracted the peptides with C18

15 reversed phase ZIPTIPs.

16 In the instantly disclosed invention, we deal with

17 proteins generally having a molecular weight of about 20 kD

18 or more. In general, proteins of greater than 20 kD can

19 reliably be fragmented by trypsin or other enzymes. The

20 instant technology incorporates sufficient sensitivity to

21 deal with even the low production of peptides from proteins

22 less than 20 kD clipped from gel.

23 Proteins differ from peptides in that they cannot be

effectively resolved by time of flight MS and they are too large (>3kD) to be effectively fragmented by collision with gases. The most commonly used solution to these problems is to resolve the proteins by polyacrylamide gel electrophoresis followed by staining with silver, or coomassie brilliant blue or rubidium dyes or counter staining with Zinc-SDS complexes. Once the proteins have been resolved and visualized with stains the proteins that differ between disease states can then be excised from the gel and the protein purified in the 1-D gel band or 2-D gel spot can be cleaved into fragments less than 3 kD by proteolytic enzymes. Once protein has been resolved by gel and cleaved by enzymes, the protein is considered in the form of peptides and therefore can be dealt with as per earlier work (09/846330). The peptide is either collected and purified with C18 reversed phase chromatography or by some other form of chromatography prior to reversed phase separation. The peptide can also be collected in ammonium carbonate buffer that is subsequently evolved by reaction with acid or by removal in organic solvents.

Once the peptides are collected they can be sequenced, e.g. with a MALDI-Qq-TOF but also with a TOF-TOF, and ESI-Q-TOF or an ION-TRAP. Other types of MS analysis which may be employed are SELDI MS and MS/MS. The peptides are

1 fragments of the original protein. The peptides are
2 sequenced by fragmentation to produced a spectrum composed of
3 the parts of the peptide. The peptide fragments can be
4 produced by a strong ionization energy with a laser,
5 temperature, electron capture, collision between the peptides
6 themselves or with other objects such as gas molecules. The
7 spacing in terms of mass between the parts of the peptides is
8 a fragmentation pattern. The fragmentation pattern of each
9 peptide from the starting mass to the last remaining amino
10 acid (from either end) is unique.

11 The human genome contains the genes that encode all
12 proteins. The proteolytic cut sites within all these
13 proteins can be predicted from the translated amino acid
14 sequence. The mass of the peptides that result from the
15 predicting cut sites can be calculated. Similarly, the
16 fragmentation pattern from each hypothetical peptide can be
17 predicted. Thus, we can conceptually digest the proteins
18 within the human proteome and fragment them.

19 When a peptide has been "sequenced" it is understood
20 that the peptide fragment has been purified by one of the
21 methods above, i.e. Time of flight (TOF) or by
22 chromatography, before fragmenting it with gas to produce the
23 peptide fragments. The original peptide mass and

1 fragmentation pattern obtained is then fit to those from the
2 theoretical digestion and fragmentation of the genome. The
3 peptide that best matches the theoretical peptides and
4 fragments and is biologically possible, i.e. a potential
5 human blood-borne protein, is thus identified. It is possible
6 to identify plural targets in this fashion.

7
8 Following are exemplary, but non-limiting examples of
9 preparatory protocols useful in the process of the instant
10 invention.

11
12 Preparatory Protocols:

13 Any of these protocols may be selected from a column
14 flow-through stream, a column elution stream, or a column
15 scrub stream.

16 Hi Q is a strong anion exchanger made of methyl acrylate
17 co-polymer with the functional group: $-N^+(CH_3)_2$;

18 Hi S is a strong cation exchanger made of methyl acrylate
19 co-polymer with the functional group: $-SO_3^-$;

20 DEAE is diethylaminoethyl which is a weak cation exchanger
21 made of methyl acrylate co-polymer with the functional group
22 $-N^+(C_2H_5)_2$;

23 PS is phenyl sepharose;

Blif
cont
Sub
B2

1 BS is butyl sepharose.

2 Note that the supports, i.e. methyl acrylate and
3 sepharose are different, but non-limiting examples, as the
4 same functional group on different supports will function,
5 albeit possibly with different effects.

6 DEAE Column Protocol:

7 1)Cast 200 µl of 50% slurry;

8 2)Equilibrate column in 5 bed volumes of 50 mM

9 tricine pH 8.8 (binding buffer);

10 3)Dissolve 25 µl of sera in 475 µl of binding buffer;

11 4)Wash column in 5 bed volumes of binding buffer;

12 5)Elute column in 120 µl of 0.4 M Phosphate buffer

13 (PB) pH 6.1;

14 6)Elute column in 120 µl of 50 mM citrate buffer

15 pH 4.2;

16 7)Scrub column with 120 µl sequentially with each

17 of 0.1% triton, 1.0% triton and 2% SDS in

18 62.5 mM Tris pH 6.8.

19
20 Butyl Sepharose Column Protocol:

21 1)Cast 150 µl bed volume column;

22 2)Equilibrate column in 5 bed volumes of 1.7 M

23 $(\text{NH}_4)_2\text{SO}_4$ in 50 mM PB pH 7.0 (binding buffer);

sub
b3
cont

~~sub
b4~~

- ~~SECRET~~

1 62.5 mM Tris pH 6.8.

2

3 HiQ Anion Exchange Mini Column Protocol:

4 1) Dilute sera in sample/running buffer;

5 2) Add HiQ resin to column and remove any air bubbles;

6 3) Add ultrafiltered (UF) water to aid in column

7 packing;

8 4) Add sample/running buffer to equilibrate column;

9 5) Add diluted sera;

10 6) Collect all the flow-through fraction in Eppendorf

11 tubes until level is at resin;

12 7) Add sample/running buffer to wash column;

13 8) Add elution buffer and collect elution in Eppendorf
14 tubes.

15

16 HiS Cation Exchange Mini Column Protocol:

17 1) Dilute sera in sample/running buffer;

18 2) Add HiS resin to column and remove any air bubbles;

19 3) Add UF water to aid in column packing;

20 4) Add sample/running buffer to equilibrate column for
21 sample loading;

22 5) Add diluted sera to column;

23 6) Collect all flow through fractions in Eppendorf

1 tubes until level is at resin;

2 7)Add sample/running buffer to wash column;

3 8)Add elution buffer and collect elution in Eppendorf
4 tubes.

5 Illustrative of the various buffering compositions
6 useful in this technique are:

7 Sample/Running buffers: including but not limited to

8 Bicine buffers of various molarities, pH's, NaCl content,

9 Bis-Tris buffers of various molarities, pH's, NaCl

10 content, Diethanolamine of various molarities, pH's, NaCl

11 content, Diethylamine of various molarities, pH's, NaCl

12 content, Imidazole of various molarities, pH's, NaCl

13 content, Tricine of various molarities, pH's, NaCl

14 content, Triethanolamine of various molarities, pH's, NaCl

15 content, Tris of various molarities, pH's, NaCl content.

16 Elution Buffer: Acetic acid of various molarities, pH's,

17 NaCl content, Citric acid of various molarities, pH's,

18 NaCl content, HEPES of various molarities, pH's, NaCl

19 content, MES of various molarities, pH's, NaCl content,

20 MOPS of various molarities, pH's, NaCl content, PIPES of

21 various molarities, pH's, NaCl content, Lactic acid of

22 various molarities, pH's, NaCl content, Phosphate of

23 various molarities, pH's, NaCl content, Tricine of various

1 molarities, pH's, NaCl content.

2 Following tryptic digestion, additional processing
3 may be carried out, for example:

4 Utilizing a type of micro-chromatographic column called a
5 C18- ZIPTIP available from the Millipore company, the
6 following preparatory steps were conducted.

- 7 1. Dilute sera in sample buffer
- 8 2. Aspirate and dispense ZIPTIP in 50% Acetonitrile
- 9 3. Aspirate and dispense ZIPTIP in Equilibration solution
- 10 4. Aspirate and dispense in serum sample
- 11 5. Aspirate and dispense ZIPTIP in Wash solution
- 12 6. Aspirate and dispense ZIPTIP in Elution Solution

13 Illustrative of the various buffering compositions
14 useful in the present invention are:

15 Sample Buffers (various low pH's): Hydrochloric acid
16 (HCl), Formic acid, Trifluoroacetic acid (TFA),

17 Equilibration Buffers (various low pH's): HCl, Formic
18 acid, TFA;

19 Wash Buffers (various low pH's): HCl, Formic acid, TFA;

20 Elution Solutions (various low pH's and % Solvents):

21 HCl, Formic acid, TFA;

22 Solvents: Ethanol, Methanol, Acetonitrile.

23 Spotting was then performed, for example upon a Gold Chip

1 in the following manner:

2 1. Spot 2 ul of sample onto each spot

3 2. Let sample partially dry

4 As a result of these procedures, the disease specific

5 markers apolipoprotein ion having a molecular weight of

6 about 1215 and a sequence ^{SEQ ID NO: 1} ~~(K)ALVQOMEQLR(Q)~~, apolipoprotein

7 ion having a molecular weight of about 1312 and a ^{SEQ ID} ~~sequence~~

8 ^{NO: 2} ~~(K)LVPFATELHER(L)~~, apolipoprotein A-IV precursor having a

9 molecular weight of about 1352.6599 and a ~~sequence~~

10 ^{SEQ ID NO: 3} ~~(R)RVEPYGENFNK(A)~~, and apolipoprotein ion having a molecular

11 weight of about 1104.5689 and a ^{SEQ ID NO: 4} ~~sequence (R)LEPYADQLR(T)~~

12 predictive of Type II diabetes.

13 Figure 1 is a photograph of a gel which is indicative

14 of the presence/absence of the marker in disease vs.

15 control and, in cases where the marker is always present,

16 the relative strength, e.g. the up or down regulation of

17 the marker relative to categorization of disease state is

18 deduced.

19 A method for evidencing and categorizing at least one

20 disease state is disclosed. The steps taken include

21 obtaining a sample from a patient, preferably human, and

22 conducting MS analysis on the sample. As a result, at least

23 one biopolymer marker sequence or analyte thereof is

1 isolated from the sample which undergoes evidencing and
2 categorizing and is compared to the biopolymer marker
3 sequence as disclosed in the present invention. The step of
4 evidencing and categorizing is particularly directed to
5 biopolymer markers or analytes thereof linked to at least
6 one risk of disease development of the patient or related to
7 the existence of a particular disease state.

8 In addition, various kits are contemplated for use by
9 the present invention. One such kit provides for
10 determining the presence of the disease specific biopolymer
11 marker. At least one biochemical material is incorporated
12 which is capable of specifically binding with a biomolecule
13 which includes at least the disease specific biopolymer
14 marker or analyte thereof, and a means for determining
15 binding between the biochemical material and the
16 biomolecule. The biochemical material for any of the
17 contemplated kits, by way of example an antibody or at least
18 one monoclonal antibody specific therefore, or biomolecule
19 may be immobilized on a solid support and include at least
20 one labeled biochemical material which is preferably an
21 antibody. The sample utilized for any of the kits may be a
22 fractionated or unfractionated body fluid or a tissue
23 sample. Non-limiting examples of such fluids are blood,

1 blood products, urine, saliva, cerebrospinal fluid, and
2 lymph.

3 Further contemplated is a kit for diagnosing,
4 determining risk-assessment, and identifying therapeutic
5 avenues related to a disease state. This kit includes at
6 least one biochemical material which is capable of ...
7 specifically binding with a biomolecule which includes at
8 least one biopolymer marker including the sequence of the
9 particular disease specific biopolymer marker or an
10 analyte thereof related to the disease state. Also
11 included is a means for determining binding between the
12 biochemical material and the biomolecule, whereby at least
13 one analysis to determine a presence of a marker, analyte
14 thereof, or a biochemical material specific thereto, is
15 carried out on a sample. As previously described,
16 analysis may be carried out on a single sample or multiple
17 samples.

18 In accordance with various stated objectives of the
19 invention, the skilled artisan, in possession of the
20 specific disease specific marker as instantly disclosed,
21 would readily carry out known techniques in order to raise
22 purified biochemical materials, e.g. monoclonal and/or
23 polyclonal antibodies, which are useful in the production of

1 methods and devices useful as point-of-care rapid assay
2 diagnostic or risk assessment devices as are known in the
3 art.

4 The specific disease markers which are analyzed
5 according to the method of the invention are released into
6 the circulation and may be present in the blood or in any
7 blood product, for example plasma, serum, cytolyzed blood,
8 e.g. by treatment with hypotonic buffer or detergents and
9 dilutions and preparations thereof, and other body fluids,
10 e.g. CSF, saliva, urine, lymph, and the like. The
11 presence of each marker is determined using antibodies
12 specific for each of the markers and detecting specific
13 binding of each antibody to its respective marker. Any
14 suitable direct or indirect assay method may be used to
15 determine the level of each of the specific markers
16 measured according to the invention. The assays may be
17 competitive assays, sandwich assays, and the label may be
18 selected from the group of well-known labels such as
19 radioimmunoassay, fluorescent or chemiluminescence
20 immunoassay, or immunoPCR technology. Extensive discussion
21 of the known immunoassay techniques is not required here
22 since these are known to those of skilled in the art. See
23 Takahashi et al. (Clin Chem 1999;45(8):1307) for a

1 detailed example of an assay.

2 A monoclonal antibody specific against the disease
3 marker sequence isolated by the present invention may be
4 produced, for example, by the polyethylene glycol (PEG)
5 mediated cell fusion method, in a manner well-known in the
6 art.

7 Traditionally, monoclonal antibodies have been made
8 according to fundamental principles laid down by Kohler
9 and Milstein. Mice are immunized with antigens, with or
10 without, adjuvants. The splenocytes are harvested from
11 the spleen for fusion with immortalized hybridoma
12 partners. These are seeded into microtiter plates where
13 they can secrete antibodies into the supernatant that is
14 used for cell culture. To select from the hybridomas that
15 have been plated for the ones that produce antibodies of
16 interest, the hybridoma supernatants are usually tested
17 for antibody binding to antigens in an ELISA (enzyme
18 linked immunosorbent assay) assay. The idea is that the
19 wells that contain the hybridoma of interest will contain
20 antibodies that will bind most avidly to the test antigen,
21 usually the immunizing antigen. These wells are then
22 subcloned in limiting dilution fashion to produce
23 monoclonal hybridomas. The selection for the clones of

1 interest is repeated using an ELISA assay to test for
2 antibody binding. Therefore, the principle that has been
3 propagated is that in the production of monoclonal
4 antibodies the hybridomas that produce the most avidly
5 binding antibodies are the ones that are selected from
6 among all the hybridomas that were initially produced.
7 That is to say, the preferred antibody is the one with
8 highest affinity for the antigen of interest.

9 There have been many modifications of this procedure
10 such as using whole cells for immunization. In this
11 method, instead of using purified antigens, entire cells
12 are used for immunization. Another modification is the
13 use of cellular ELISA for screening. In this method
14 instead of using purified antigens as the target in the
15 ELISA, fixed cells are used. In addition to ELISA tests,
16 complement mediated cytotoxicity assays have also been
17 used in the screening process. However, antibody-binding
18 assays were used in conjunction with cytotoxicity tests.
19 Thus, despite many modifications, the process of producing
20 monoclonal antibodies relies on antibody binding to the
21 test antigen as an endpoint.

22 The purified monoclonal antibody is utilized for
23 immunochemical studies.

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1 the like, to diagnose patients with the characteristic
2 disease state linked to said marker sequence. The
3 antibodies can be used in any type of immunoassay. This
4 includes both the two-site sandwich assay and the single
5 site immunoassay of the non-competitive type, as well as
6 in traditional competitive binding assays.

7 Particularly preferred, for ease and simplicity of
8 detection, and its quantitative nature, is the sandwich or
9 double antibody assay of which a number of variations
10 exist, all of which are contemplated by the present
11 invention. For example, in a typical sandwich assay,
12 unlabeled antibody is immobilized on a solid phase, e.g.
13 microtiter plate, and the sample to be tested is added.
14 After a certain period of incubation to allow formation of
15 an antibody-antigen complex, a second antibody, labeled
16 with a reporter molecule capable of inducing a detectable
17 signal, is added and incubation is continued to allow
18 sufficient time for binding with the antigen at a
19 different site, resulting with a formation of a complex of
20 antibody-antigen-labeled antibody. The presence of the
21 antigen is determined by observation of a signal which may
22 be quantitated by comparison with control samples
23 containing known amounts of antigen.

1 Antibodies may also be utilized against the disease
2 specific markers, as haptens, to create an antibody
3 response against the protein to which it binds, thereby
4 identifying targets for treatment of the disease or a sub-
5 class thereof.

6 Lastly, the markers and associated antibodies provide
7 a tool for monitoring the progress of a patient during a
8 therapeutic treatment, so as to determine the usefulness
9 of a novel therapeutic agent.

10 All patents and publications mentioned in this
11 specification are indicative of the levels of those
12 skilled in the art to which the invention pertains. All
13 patents and publications are herein incorporated by
14 reference to the same extent as if each individual
15 publication was specifically and individually indicated to
16 be incorporated by reference.

17 It is to be understood that while a certain form of
18 the invention is illustrated, it is not to be limited to
19 the specific form or arrangement herein described and
20 shown. It will be apparent to those skilled in the art
21 that various changes may be made without departing from
22 the scope of the invention and the invention is not to be
23 considered limited to what is shown and described in the

1 specification and drawings/figures.

2 One skilled in the art will readily appreciate that
3 the present invention is well adapted to carry out the
4 objectives and obtain the ends and advantages mentioned,
5 as well as those inherent therein. The oligonucleotides,
6 peptides, polypeptides, biologically related compounds,
7 methods, procedures and techniques described herein are
8 presently representative of the preferred embodiments, are
9 intended to be exemplary and are not intended as
10 limitations on the scope. Changes therein and other uses
11 will occur to those skilled in the art which are
12 encompassed within the spirit of the invention and are
13 defined by the scope of the appended claims. Although the
14 invention has been described in connection with specific
15 preferred embodiments, it should be understood that the
16 invention as claimed should not be unduly limited to such
17 specific embodiments. Indeed, various modifications of the
18 described modes for carrying out the invention which are
19 obvious to those skilled in the art are intended to be
20 within the scope of the following claims.

21